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(54) Title: CHEMILUMINESCENT TEST FOR MICROORGANISMS

(57) Abstract

The invention provides a method and reagent composition for the detection or characterization of microorganisms by the emission of light. The composition includes a substrate capable of metabolism by viable microorganisms of interest and a chemiluminescent probe and may also include a redox active agent. The method may be used to detect or to characterize bacteria, fungi or yeast in diverse samples from the environment, industry, food, veterinary or medical sources. The assay composition and method is particularly useful to detect bacteriuria.

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CHEMILUMINESCENT TEST FOR MICROORGANISMS

FIELD OF THE INVENTION

The field of the invention is test compositions and methods for the detection, quantitation and characterization of viable microorganisms in general and chemiluminescent test compositions and methods for the detection and characterization of viable microorganisms by the measurement of emitted light, in particular.

BACKGROUND OF THE INVENTION

Extensive efforts have been devoted to detection of microorganisms in specimens by use of bioluminescence. In general, two types of bioluminescence systems have been described. One system measures adenosine triphosphate (ATP) which has been extracted from the microorganisms in the sample. ATP at levels as low as 10^{-14} M may be measured with the firefly luciferin-luciferase system. The assay procedure involves disrupting the microorganisms in the sample to release intracellular ATP. The extract is added to the luciferin-luciferase reagents and if ATP is present, a burst of light is emitted within a few seconds. This method measures the ATP present in cells at the time of extraction and does not take advantage of the amplifying power of the enzymes that produce ATP. An additional disadvantage is that luciferase is expensive, unstable and is inhibited by a wide variety of compounds, including salts.

The second bioluminescence system measures NAD and NADP extracted from the cells in the sample or specimen. Following extraction for these cofactors, a dehydrogenase and its substrate, such as alcohol dehydrogenase and

ethanol, are added. This reaction mixture is then added to riboflavin reductase, an aldehyde and luciferase from the marine bacterium Photobacterium fischeri. If the reduced cofactors are present, a burst of light is emitted within a few seconds. Again, this system does not take advantage of the amplifying power of cellular dehydrogenases. In addition it requires extra reagents and an incubation step for the extracellular reduction of the cofactors.

Both the bioluminescence methods discussed above use enzyme reagents to measure cofactors of cellular reactions. Since enzymes are macromolecular and cannot penetrate cell walls, the cofactors must be extracted from the cells and added to the bioluminescence reagents. The extraction adds cumbersome steps to the assay, complicating the procedure. The cell walls must be disrupted by methods that do not leave residues in the extract which residues would interfere with the assay. In contrast, the method of the present invention preferably uses low molecular weight reagents that can penetrate cells and cell disruption is not necessary and not desirable.

Another method for testing for bacteria, reacts the specimen with a peroxide, such as sodium perborate or sodium pyrophosphate peroxide, and luminol in 0.04 to 0.12 M sodium hydroxide. [Oleniacz, W.S., et al., Environ. Sci. Technol. 2:1030-1033 (1968)] Hematin from the cells catalyzes the oxidation of luminol by peroxide. Light emission occurs as a burst that lasts about 30 seconds. In principle the hematin catalyzes the oxidation of the luminol but under the strongly alkaline reaction conditions, hematin decomposes quickly in the presence of peroxides. Therefore the hematin catalyst turns over only once or, at the most, a few times. Thus, this system achieves very little amplification. In addition, this assay is ineffective for detection of bacteria, including most anaerobic species, that do not

produce hemin. The specificity of the assay is diminished because iron salts which are found in many types of specimens also catalyze the alkaline oxidation of luminol by peroxides. [Ewetz, L. and Thore, A., Anal. Biochem., 71:564 (1976)].

Polymorphonuclear leukocytes (PMNL) stimulated by specific substances such as opsonified zymosan emit substantial levels of light due to the production of superoxide ion by a unique NADPH oxidase present in these leukocytes. The light emission can be increased 1000 fold by incubating the cells with the probes lucigenin or luminol. [Allen, R.C. in (Adam, W. and Cilento, G., eds) Chemical and Biological Generation of Excited States, Academic Press, N.Y. pp 309-344 (1982)] Allen uses PMNL luminescence response to opsonified zymogen as an example to illustrate the mechanism of chemiluminescence. In Figure 5 he shows data related to this response as followed by use of a barbitol buffer containing calcium and magnesium ions, 0.1 g/dl glucose and albumin at pH 7.2 and zymogen opsonified with fresh autologous serum. Light emission from leukocytes with or without the use of the chemiluminescent probes is specific for activation of the cells by extraneous agents such as opsonified zymosan and has not been used to detect the presence of cells in an unknown sample.

Peters et al. studied lucigenin chemiluminescence as a probe for measuring reactive oxygen species production in Escherichia coli [Peters et al., Analytical Biochemistry, 186, 316-319 (1990)]. It was found that the addition of 100% oxygen to a whole cell suspension of E. coli in the presence of lucigenin produced an increase in light emission of 200% over the control. Redox reagents such as paraquat and menadione, known to increase intracellular production of O_2^- and H_2O_2, also caused a measurable increase in lucigenin chemiluminescence. The chemiluminescence produced with E. coli cells and lucigenin in air was less than in 100%

oxygen and heat killed cells showed no increase in chemiluminescence on addition of either oxygen, paraquat or menadione. Experiments were carried out on cells grown aerobically in LB medium supplemented with 0.5% glycerol, and with LB medium supplemented with 0.3% xylose. Harvested cells were suspended in glycerol taxis buffer (GBT) which contained 100 μM EDTA, 10 mM potassium phosphate buffer, 1 mM magnesium sulfate, 1 mM ammonium sulfate and 10 mM glycerol (final pH 7.0). One (1) ml aliquots of the cell suspension were treated with lucigenin to provide a final lucigenin concentration of 40 μM. Samples were placed in a luminometer and the chemiluminescent response to the addition of oxygen or air was measured. Peters et al. concluded that lucigenin may be used as a chemiluminescent probe to demonstrate continuous intracellular production of reactive oxygen metabolites in E. coli.

Nakano et al. [Analytical Biochemistry, 187, 227-280 (1990)] discloses a method for determining superoxide dismutase in various tissues and blood cells with cypridina luciferin analogs, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-α]pyrazin-3-one and 2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2-α]pipazine-3-one (abbreviated MCLA and CLA respectively).

SUMMARY OF THE INVENTION

The invention provides a chemiluminescent reagent composition useful for the detection or characterization of viable microorganisms, comprising a substrate capable of metabolism by viable microorganisms of interest to produce reducing equivalents; and a chemiluminescent probe. Optionally the composition may also contain a redox active agent. Preferred compositions include lucigenin and the substrate; and luminol, a redox active agent and a substrate.

The invention also provides a method of detecting or characterizing viable microorganisms, comprising the steps of: contacting a sample suspected of contamination with microorganisms of interest with a chemiluminescent reagent composition comprising a substrate capable of being metabolized by the microorganism of interest and a chemiluminescent probe; and measuring the light. The method may also be used for characterizing viable microorganisms by utilizing a substrate characteristic for the metabolism of the microorganisms of interest. The method may include pretreatment of the sample to provide a concentrate containing the microorganisms of interest in which case the concentrate is contacted with the reagent composition. Pretreatment may be accomplished, for example, by filtering or centrifuging.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Conventional culture methods for detection of microorganisms, such as bacteria, yeast and fungi, require several hours to several days incubation before results become available. Often the need to know whether a sample is contaminated with microorganisms passes before results from culturing become available. The present invention gives results within a few minutes. The method produces light which appears quickly and can be measured at very low levels with commercial luminometers. Therefore the method provides a useful screening test for low levels of microorganisms. A screening test is especially useful when a high proportion of samples are not contaminated. Then only positive samples need to be processed further if the identity of the contaminants is important.

Chemiluminescent reagent compositions and a method for the detection and quantification of microorganisms are provided. The method may be used to detect or characterize microorganisms including bacteria, fungi or

yeast depending on the substrate used. The method diverts electron flow generated by cellular metabolic processes into chemiluminescent reactions. The light generated may be detected by placing the sample in a luminometer and measuring light emission.

The assay uses the chemical reducing activity of cell metabolism to generate chemiluminescence. Oxidation-reduction reactions occur in all living cells. Therefore, the assay may be applied to the detection of a wide variety of microorganisms. These include prokaryotic and eukaryotic microorganisms that grow under aerobic and/or anaerobic conditions. Examples are bacteria, fungi, algae, plankton and protozoa. Organisms containing significant levels of pigment could be detected, but the detection limits would be higher unless the light emitted were measured over a longer period or additional components were added to the reagent composition to increase the light produced.

The chemiluminescent method of the invention diverts electron flow generated by cellular metabolism into chemiluminescent reactions. Reducing equivalents occur in the form of reduced cofactors including reduced NAD, reduced nicotinamide adenine dinucleotide phosphate (NADP), reduced flavins, reduced ubiquinones, reduced menaquinone, reduced pyrroloquinoline quinone, reduced cytochromes, and so forth. The assay conditions should favor the activity of enzymes that reduce such cofactors because reducing equivalents are diverted from the cofactors to chemiluminescent reactions. In some situations, exogeneous cofactors can be included with the assay reagents to increase light production. For example, several species of bacteria produce apoglucose oxidases that can be activated by exogeneous pyrroloquinolone quinone. [Van Schie, et al., J. Bacteriol., 163:493 (1985)]

CHEMILUMINESCENT COMPOSITIONS

The chemiluminescent reagent compositions of this invention are generally composed of a substrate capable of metabolism by the viable microorganisms of interest to produce reducing equivalents and a chemiluminescent probe. Depending on the reaction system, an additional component, a redox active agent may also be included. Other components, such as buffers and enzyme cofactors, may be included depending on the conditions optimal for metabolism in the microorganism of interest. However, it is not necessary to use reagents such as opsonified zymogen, perborate or peroxide.

Substrates

A variety of substrates can be catabolized by microorganisms and those that yield reducing equivalents are potentially useful for the present assay. The substrate is chosen depending on the metabolism of the microorganism of interest and will depend on the purpose of the assay. For example a screening test designed to detect a wide variety of microorganisms should employ a substrate such as glucose which can be metabolized by many species of microorganisms. In some situations it can be advantageous to use several substrates in a cocktail in order to broaden the range of detectable microorganisms. Cocktails can also be used advantageously to activate several metabolic pathways simultaneously and increase the chemiluminescence due to the increased electron flow. In general, glucose, citrate or mixtures thereof are suitable substrates to detect the presence or absence of bacteria. Some other useful substrates are glycerol, succinate, acetate, lactate, pyruvate and galactose. Others may be identified by those of skill in the art from substrates

for the metabolic reactions of the microorganisms of interest.

In other situations it will be desirable to detect only microorganisms that can metabolize a specific substrate. For example, ethanol is a suitable substrate for yeast and its use can differentiate between the presence of a bacterial contaminant such as E. coli and yeast since E. coli does not metabolize ethanol. Bacteria and fungi are used to degrade waste chemicals in waste water plants. The efficiency of such plants is controlled by monitoring the level of microorganisms that oxidize specific compounds such as phenols. Therefore, such monitoring can be accomplished by means of the composition and method of this invention when phenols are used as the substrate.

Chemiluminescent Probes

Chemilumigenic compounds of principal interest as probes in the present invention are the aminophthalhydrazides as exemplified by luminol and dimethylbiacridylium salts such as lucigenin. Other chemiluminescent probes such as MCLA or CLA may also be used as equivalents to lucigenin. At the neutral to mildly alkaline pH used for the detection of viable microorganisms, aminophthalhydrazides undergo dioxygenation by adding the elements of hydrogen peroxide to produce light, nitrogen and aminophthalates. Dimethylbiacridylium salts undergo reductive dioxygenation which forms an unstable dioxatane. The dioxatane decomposes spontaneously to produce light and N-methylacridone.

Dimethylbiacridylium salts and aminophthalhydrazides are activated in chemiluminescent reactions by different molecular mechanisms. The optimum conditions for the assay will be somewhat different when each class of compounds is used as a probe. Because it is not unusual

for the assay reagents to emit some background light in the absence of cells, reaction conditions are typically optimized to obtain the highest signal/background ratio. If the level of background light is acceptable, the chemiluminescent compounds are used in excess so the maximum rate of light emission is achieved.

Luminol is a preferred probe. Derivatives and analogs such as isoluminol are also useful. In particular the addition of alkyl groups on the amino group of luminol, isoluminol and naphthalhydrazides provides derivatives which produce light in high quantum yields [Gundermann, K.D., Angew. Chem. internat. Edit. 4:566-573 (1965), Brundrett, R.B. et al., J. Amer. Chem. Soc., 94:7536-7541 (1972)]. Other derivatives, known to those of skill in the art of chemiluminescence, are also known to produce light in high yields and may be used in the reagent composition.

When biacridylium salts such as lucigenin (10, 10'-dimethyl-9,9'-biacridylium nitrate) are used, redox active agents are not essential for chemiluminescence. However, the addition of low levels of redox active agents such as menadione, 1-methoxyphenazine methosulfate, plumbagin or phenothiazines such as Azure C and methylene blue may be used to increase chemiluminescence. Azure C is a preferred redox active agent for use with lucigenin in concentrations of about 0.4 to 2.0 mM lucigenin. The concentrations of redox active agents that give maximum light yields depends on the agent used; however, the optimum concentrations have been found to occur in the range of 5 to 100 μM.

Lucigenin is reduced by cellular enzymes [Greenlee, L., et al., Biochem. 1:779-783 (1962)] and in this form can react with the low levels of superoxide ion normally produced in cells. The dioxatane produced by this sequence of reactions decomposes to emit light as discussed above.

Lucigenin concentration will usually be chosen to maximize light emission in the presence of microorganisms and minimize background light in the absence of cells. The optimal concentration for a particular situation can be determined experimentally by one skilled in the art. Light emission with lucigenin can be increased by including a redox active agent that will react in its reduced form to produce superoxide ion. Reagent concentrations in such an assay should be co-optimized to balance lucigenin and redox active agent concentrations versus superoxide ion production to achieve maximum light yield. Experimental co-optimization of reagents is preferred because reagents become partitioned between intra- and extracellular spaces and interact at various rates with different cell enzymes.

Redox active agents

For the purposes of the present invention, redox active agents are defined as compounds which can receive electrons from the electron transport systems of the microorganisms to be detected and can transfer the electrons to molecular oxygen and/or chemiluminescence compounds. These compounds are in the oxidized state when they are added to the sample, since the assay method is aerobic. Redox active agents preferred in the invention are those capable of penetrating into microbial cells. Redox active agents will be included in the reaction mixture at levels that maximize light emission. The concentration of colored redox agents might have to be limited to avoid quenching of emitted light.

The intracellular redox activities of a large number of compounds were studied by Hassan and Fridovich, Arch. Biochem. Biophys., 196:385-395 (1978). Compounds which are able to scavenge reducing equivalents from normal electron transfer pathways are suitable for use in this invention. Examples include paraquat, pyocyanine,

phenazine methosulfate, streptonigrin, juglone, menadione, plumbagin, methylene blue and azure C. The concentrations of redox active agents will be optimized experimentally to maximize chemiluminescence. Reactions between reduced redox active agents and oxygen are reversible and equilibrium conditions will depend on the reduction potentials of the reactants in addition to factors such as pH [Afamas'ev, I.B., et al., Arch. Biochem. Biophys., 281:245-250 (1990)]. The partitioning of redox active agents between intra and extracellular solutions and the variable activities of the agents with different cellular enzymes makes prediction of optimal reagent concentrations uncertain. Therefore, experimental optimization is preferable.

Other components

The reagent composition may also contain other components such as buffers to control pH. A buffer capable of providing a pH between about pH 6 and about pH 10 is preferably included in the composition. The buffer salt and pH should be optimized to minimize reagent background light while maximizing light generated specifically by cellular metabolism. Chemiluminescent reactions compete with physiological oxidants for reducing equivalents generated by cell enzymes. The physiological oxidant for aerobic microorganisms is oxygen and it cannot be removed conveniently from reaction mixtures. However, oxygen is reduced by cytochrome oxidase at the termini of electron transport chains. Inhibitors can be used to intercept electron flow to oxygen. Alternatively, electron transport to oxygen can be slowed by depriving the cells in the assay medium of factors, such as magnesium ion and phosphate, which are required for oxidative phosphorylation. Since oxidative phosphorylation and reduction of oxygen are coupled, deprivation of essential factors will slow

electron flow to oxygen and cause accumulation of a larger pool of reducing equivalents for diversion to chemiluminescent reactions.

ASSAY METHOD

Preparing the sample

Samples may be taken from any medium of interest. Commonly such samples will be from environmental, industrial, food, veterinary, or medical sources. Specimens from human and animal sources include urine, whole blood, plasma, serum, amniotic fluid, cerebrospinal fluid, sputum, fecal matter, throat swabs and genital swabs.

The sample processing procedure should be adjusted for each type of sample. In general, however, the processing should not be deleterious to the metabolic functions of microbial cells. If the sample is liquid and the suspected level of microorganism contamination is below the assay sensitivity, it may be useful to concentrate the cells by centrifugation, filtration or other means available to those of skill in the art. The cell pellet or filtered cells may then be combined with the chemiluminescent reagents for assay. Centrifugation or filtration may also be useful for separating sample components which could interfere with the chemiluminescent reactions. For example, a sample may contain colored matter that absorbs chemiluminescence. In addition, the chemiluminescent probes and redox active agents compete with physiological oxidants for electrons passing along electron transport chains. Removal of physiological oxidants provides a larger pool of electrons for the chemiluminescent reactions and increases the sensitivity of the assay. In the case of anaerobic microorganisms the physiological oxidants can be substances like nitrate and sulfate. Sample

processing methods such as centrifugation and filtration can be used to remove such oxidants before the sample is combined with assay reagents.

Adding the reagent composition

The concentration of all the components of the chemiluminescent test composition will be chosen to maximize light emission from the cells of interest and to minimize background light. However, the examples herein provide one of skill in the art with guidelines for such concentrations.

The reagent compositions of this invention will usually be combined with the sample a short time before the light emission is measured. Control of the reaction temperature will often be beneficial to optimize light output and reproducibility. The temperature will typically be between about 10 and 40° C, but higher temperatures may be employed especially for the detection of thermophilic microorganisms.

Small reaction volumes are preferred. Often sample volumes will be small, either from processing as described previously or simply because of the nature of the sample as, for example, with a throat or genital swab sample. In addition, the temperature of small volumes may be adjusted more readily and small reaction containers may be positioned closer to a photodetector for efficient light detection. Furthermore, reagent concentrations will be more critical to assay performance than the quantity of reagents per assay. If the reagents emit background light in the absence of cells, the use of small reaction volumes will minimize this background signal.

The reaction mixture will contain one or more substrates. The substrates will be chosen to accommodate the types of microorganisms to be detected. Substrates

will commonly be added in excess. If the substrates contribute to background light, the concentration used might need to be limited to achieve the best signal to background ratio. Substrate choice and the concentration of use thereof will be well within the skill of those versed in the art of chemiluminescence measurement given the guidance in the examples.

In some situations, the concentration of the chemiluminescent probe could be limited by the solubility of the compound. In virtually all situations, however, only a small portion of the probe in the reaction mixture will be consumed during the reaction and therefore the solubility limitation is usually not a problem.

To take advantage of the amplifying power of the cell enzymes, the assay should be conducted under physiological conditions with regard to temperature, pH and reaction medium. It is also advantageous to carry out the assay with intact cells so enzyme cofactors and substrates are maintained at physiological concentrations. All assay reagents have low molecular weights. Because of this, they can penetrate the cell walls of a variety of microorganisms and function in the intracellular environment.

Measuring

The assay may be conducted with a cell suspension in a solution of the assay reagents. A test tube containing the reaction mixture would be positioned near a photodetector or photographic film for measurement of emitted light. Variations on this method are also feasible. For example, if microorganisms are collected on a swab, the swab can be saturated with a solution of assay reagents and positioned near the light detection system. If cells are collected on a porous membrane or fiberous filter, the membrane or filter may be wetted

with a reagent solution and mounted near the light detection system.

Light emission can be measured electronically with a luminometer using photon counting if high assay sensitivity is required. The light may also be measured by exposing photographic film to reaction mixtures. Clouding of developed film will indicate the level of light emitted. The light intensity may be compared to a calibration scale to estimate the number of microorganisms present. If a qualitative determination of contaminated versus uncontaminated samples is adequate, the light intensity can be compared to a predetermined standard to provide a yes/no answer.

Chemiluminescence from these assays persists for many minutes, sometimes for hours. The duration of light measurements may be adjusted to meet performance requirements. In general, light emission will be measured for an adequate period to give statistically significant data, usually from about 0.5 to about 5 minutes. If assay time is not a limiting factor, luminescence can be measured for much longer periods, up to a few hours. A longer measuring period increases assay sensitivity if background light is low or decreases with time.

USES

The assay may be used for the detection of both aerobic and anaerobic microorganisms and may be used for the characterization of microorganisms. For instance, if a microorganism has a distinguishing ability to metabolize a substrate by a mechanism that yields reducing equivalents, the electron flow can be diverted into chemiluminescent reactions and measurement of emitted light is an indicator of a characteristic metabolic capability. Conversely, the present assay may be used to evaluate the ability of a microorganism to

grow and multiply in a medium containing a characteristic metabolite. A selective medium containing the metabolite is inoculated with the sample and the cell population is monitored periodically by assaying aliquots of the culture medium by the chemiluminescent method.

The assay may also be applied to medical diagnostics. For example, urinary tract infections are diagnosed by the presence of a characteristic number of microorganisms per milliliter of urine. Screening tests are used to identify infected specimens. The assay method may also be used in agriculture and food industries where the discovery of microbial contamination of products is important.

The assay may also be used to determine antibiotic sensitivity of infective microorganisms. A sample, for example from a throat swab, would be contacted with the chemiluminescent reagent composition and light measured. A second sample would be contacted with the chemiluminescent reagent composition which additionally includes an antibiotic, and light measured. A differential in the light emitted by the control and the antibiotic added sample, would indicate sensitivity of the infecting microbes to the antibiotic tested.

Environmental applications may include testing of river, lake and well waters for microbial content. The chemiluminescent method of the present invention could also be used to assay for environmental contaminants such as heavy metals. A microorganism whose metabolism is inhibited by the contaminant would be combined with the environmental sample along with the chemiluminescense reagents. The light emission from this mixture would be compared to light emitted by a control without the environmental sample. A decrease in light from the test mixture compared to the control would indicate the presence of the contaminant in the sample.

The assay could also be used to determine if a toxicant is present in a sample. The sample suspected of

containing a toxicant would be contacted with a microorganism capable of being affected by the toxicant, or a cocktail of microorganisms, and chemiluminiscent reagent composition added. The light produced is then measured and compared to a control or standard curve to determine any differential in the light produced.

ADVANTAGES

The invention has several advantages over the previous chemiluminescence and bioluminescence assays. First, the assay reagents are relatively inexpensive. Second, the reagents are low molecular weight and therefore the cells in the sample do not have to be disrupted for the assay. Third, the enzymes involved in electron transport provide a steady flow of electrons to the chemiluminescent reactions thus providing amplification. Also, the steady-state light emission allows the assay method to be carried out by conventional pipetting techniques and so forth. In comparison, bioluminescence assays require rapid mixing of assay reagents with sample because the light burst occurs a few seconds later. Such rapid mixing can only be achieved with special equipment that adds to assay cost.

Particular embodiments of the invention are illustrated in the examples. However, the invention is not limited by such examples, which are solely defined by the claims.

EXAMPLES

Culture of Microorganisms

The following microorganisms were obtained from the American Type Culture Collection: E. coli (ATCC 23716), Bacillus subtilis (ATCC 29056), Staphylococcus aureus (ATCC 25923) and Sacchromyces cerevisiae (ATCC 834). In

addition, a bacterium tentatively identified as E. coli was isolated from a farm yard sample.

Bacteria were grown to mid-log phase at 37°C in TSY broth composed of 36 g tryptone soy broth and 6 g yeast extract per liter. Cells were collected by centrifugation and resuspended in 50 mM sodium phosphate buffer, pH 7.0, at a cell density giving an absorbance at 600 nm of about 1.3. Cell counts were determined by diluting the suspensions in TSY broth and mixing aliquots of the dilutions with melted agar (40° C) containing 5 g tryptone, 2.5 g yeast extract, 1 g glucose and 16 g agar per liter. The agar plates were incubated at 37° C for 2 days and colonies were counted.

Yeast were grown at 25° C in a liquid medium containing 15 mM KH_2PO_4, 4.6 mM K_2HPO_4, 0.4 µM $CuSO_4$, 2.8 µM $Fe(NH_4)_2(SO_4)_2$, 1 mM $MgSO_4$ 6.2 µM $ZnSO_4$ plus 10 g glucose, 5 g tryptone and 1 g yeast extract per liter. Cells were collected by centrifugation and resuspended in 50 mM sodium phosphate buffer, pH 7.0. Cell counts were determined by diluting cell suspensions and mixing aliquots with melted (40°C) solid agar. The solid agar was prepared by adding 160 g agar to the liquid medium described above. The agar plates were incubated at 25°C for 2 days and then colonies were counted.

Chemiluminescence Measurements

Chemiluminescence was measured at ambient temperature with a Turner Designs 20e luminometer. One milliliter reaction mixtures were prepared at room temperature.

Typically, the cell suspension was omitted from reaction mixtures and background light was measured for 2 minutes. Then the cell suspension was added and light emission was measured for another 2 minutes. In cases where background light emission changed with time,

background light was measured with one solution and a fresh mixture was prepared to measure light produced with cells. Light production is expressed in arbitrary units.

Buffers used were 50 mM sodium phosphate, pH 6.0 and 7.0, 50 mM Tris-hydrochloride, pH 8.0 and 9.0, and 50 mM sodium carbonate, pH 10.0. Other solutions were 1 M glucose, 100 mM paraquat, 100 mM juglone in acetone, 100mM plumbagin in acetone, 100 mM sodium citrate, pH 8.9, 100 mM sodium azide, 10 mM magnesium chloride.

Example 1. Chemiluminescense with E. coli

Measurements with Lucigenin

Reaction mixtures were prepared with 0.5 mM lucigenin, 100 mM glucose, 10^8 bacteria cells (the environmental isolate) and various buffers at pH 5 to pH 10. Significant light was produced at pH 6.0 and the intensity increased as the pH was increased. Maximum light was emitted with 50 mM Tris-hydrochloride buffer, pH 9.0. At the optimum pH, the light emitted for a 2 minute period was 19.2 units and the reagent background was 0.13 unit.

Magnesium chloride was included at 0.2 mM in a reaction mixture and the light production was decreased by 67%. At 0.5 mM magnesium chloride, the light was reduced by only 57%.

A reaction mixture prepared with 50 mM sodium phosphate buffer, pH 7.0, had a reagent background light production of 0.007 unit and light emission increased to 4.74 units with 10^7 cells. Sodium azide included at 7.5 mM in a reaction mixture decreased the light production by 82%.

Light emission by E. coli (ATCC 23716) was measured with 50 mM Tris-hydrochloride, pH 8.9, containing 0.5 mM magnesium chloride. Reaction mixtures containing 0.5 mM lucigenin, 100 mM glucose and the following levels of cells gave the indicated light production:

Cells/Assay	Light Emission
0	0.12
2.5×10^6	0.19
2.5×10^7	1.35
1.0×10^8	4.96

Measurements with luminol

The following experiments were conducted with 100 µL of 5.6 mM luminol (in 50 mM Tris-hydrochloride buffer, pH 9.0), 10 µL of 100 mM paraquat and 100 µL of 1 M glucose, 100 µL of cell suspension (10^9 cells per milliliter, environmental isolate) and buffers at various pH to give 1 mL final volume. Significant light was emitted at pH 9.0 and 10.0. At pH 9.0 the reagent background light was 0.002 unit and light produced by 10^8 cells was 0.51 unit. The intensity of the light remained virtually constant for at least 10 minutes indicating that light production was dependent on steady-state processes. Light was not produced by cells in the absence of paraquat.

Replacement of paraquat with juglone or plumbagin was tested using 50 mM sodium carbonate buffer, pH 10. Reagent background light with 0.5 mM juglone was 0.45 unit and a reaction with 10^8 cells emitted 2.9 units. Similarly reagent background light with 0.5 mM plumbagin was 0.33 unit and 10^8 cells emitted 0.86 unit.

Example 2. Chemiluminescence with Bacillus subtillus

Measurements with lucigenin

A suspension of B. subtilis containing 6.7 x 10^8 cells per milliliter was used for the following studies. The reaction buffer was 50 mM Tris-hydrochloride buffer, pH 9.0, containing 0.5 mM magnesium chloride. A reaction mixture containing 0.5 mM lucigenin and 100 mM glucose gave reagent background light of 0.11 unit. Then 6.7 x 10^7 cells were added and the light emission was integrated for successive 2 minute intervals to give the following results:

Two Minute Integration Period	Light Emitted
1st	0.76
2nd	0.92
3rd	0.87
4th	0.73

These results show that light emission is a steady-state process. When cells were added to a reaction mixture containing 0.5 mM lucigenin but no glucose the net light produced was 0.11 unit. Then glucose was added and the net light production increased 5.6 fold to 0.63 units. Evidently endogeneous cell substrates can drive light production, but there is a significant increase due to the cellular metabolism of glucose.

Example 3. Luminescence Generated with S. aureus

Experiments with lucigenin

A suspension of S. aureus in 50 mM sodium phosphate buffer, pH 7.0, containing 4.4 x 10^8 cells per milliliter was used for these studies. First, reaction mixtures containing 0.5 mM lucigenin, 100 mM glucose and 4.4 x 10^7 cells were prepared with various buffers from

pH 6.0 to pH 10.0. The most intense light emission occurred at pH 10. The reagent background light was 6.0 units. With 4.4 x 10^7 cells per milliliter at pH 10, the following levels of light were measured for successive two minute integration periods:

Two minute Integration Period	Light Emitted
1st	80
2nd	132
3rd	170
4th	181
5th	168
6th	157

When 1.0 mM magnesium chloride was included in the above reaction mixture, light production was decreased by 49%. Also, 10 mM sodium azide inhibited light production by 89%.

Sodium citrate was also found to be an effective substrate for obtaining light emission with lucigenin. One hundred microliters of 100 mM sodium citrate, pH 8.9, was added in place of glucose, to the reaction mixtures described above. Two minute integrated light emission for this mixture as well as the emission from endogeneous substrate (no added substrate) were as follows:

Two Minute Integration Period	Light Emission	
	endogeneous	citrate
1st	3.6	6.4
2nd	5.6	11.0
3rd	6.9	14.6

Light emitted with citrate was substantially higher than that generated by endogeneous substrates.

Studies with luminol

This work employed 1.0 mL reaction mixtures made up with 100 µL of 10 mM luminol (in 50 mM sodium carbonate buffer, pH 10.0), 100 µL 1 M glucose, 5 µL of a redox active agent, 745 µL of 50 mM sodium carbonate buffer, pH 10.0 and 100 µL of cell suspension containing 4.4 x 10^8 cells per milliliter. A mixture containing 5 µL of 100 mM plumbagin but no cells produced reagent background light of 0.93 units. Addition of cells increased the light production to 3.94 units. Plumbagin and luminol were both found to be essential for light production with this reaction mixture.

A reaction mixture containing 5 µL of 100 mM juglone produced reagent background light of 0.65 units. When cells were included, the light yield increased to 1.74 units.

Example 4. Chemiluminescence Generated with Yeast

Studies with lucigenin

A suspension of Saccharomyces cerevisiae in 50 mM sodium phosphate, pH 7.4, and with an absorbance at 600 nm of 3.06 was used for the following measurements. Reaction mixtures were prepared with 100 µL of 5 mM lucigenin, 100 µL 1 M glucose, 100 µL of the cell suspension and 700 µL of buffers at various pH. The optimum condition for light production was 50 mM Tris-hydrochloride, pH 9.0. The reagent background light was 0.091 unit and the light emission with cells present was 1.74 units. A reaction mixture containing cells, but no

glucose, emitted 0.6 unit light due to endogeneous cell substrates.

A reaction mixture was prepared with 50 µL ethanol in place of the glucose. The following light emission was measured for successive two minute integration periods. The light emitted due to endogeneous substrates is also presented for comparison.

Two Minute Integration Period	Light Emission	
	Endogeneous	Ethanol
1st	0.60	2.68
2nd	0.95	5.78
3rd	1.33	9.11

The results show that ethanol can serve as a substrate for the chemiluminescence.

Measurements with luminol

Reaction mixtures were prepared with 100 µL 10 mM luminol (in 50 mM sodium carbonate buffer, pH 10), 100 µL 1 M glucose, 10 µL 100 mM paraquat, 100 µL of cell suspension and 690 µL 50 mM sodium carbonate, pH 10.0. Light emission from this reaction and one without the cell suspension (reagent control) was integrated during successive two minute intervals:

Two Minute Integration Period	Light Emission	
	Reagent Control	Cells
1st	4.29	5.68
2nd	5.50	9.25
3rd	6.36	14.5
4th	7.1	16.4

The reaction mixtures with cells emitted substantially more light than the control. Glucose and paraquat were required for light production by the cells.

Five microliters of 100 mM juglone was included in a reaction mixture in place of paraquat. Light emission in the absence of cells was 1.05 units and in the presence of cells was 2.27 units. Thus, juglone also functioned as a redox active agent in this system.

Example 5. Luminescence generated with E. coli, lucigenin and various redox active agents.

The experiments described below utilized a BioOrbit 1251 luminometer and light emission from reactions was monitored for one minute in arbitrary units displayed on the luminometer. Reactions used 2.8 X 10^7 E. coli cells per assay (farm yard isolate) and were conducted in a total volume of 0.5 mL at ambient temperature.

Menadione as redox agent

A series of reactions were conducted with 50 mM sodium pyrophosphate buffer, pH 9.0, and variation of menadione concentration showed that maximum light emission occurred with 0.02 mM menadione. The variation of lucigenin showed that the optimum concentration was 0.2 mM. Variation of glucose concentration between 0.4 and 5.0 mM did not have a substantial effect on light emission. There was not significant light emission when glucose was omitted from the reaction mixture.

Reactions were conducted with or without 0.2 mM menadione in 50 mM Bis Tris Propane buffer, pH 9.5, 2.5 mM glucose and 0.2 mM lucigenin. In the absence of menadione the light emission was 645 and in the presence of menadione it was 7250 demonstrating a more than ten fold increase in light emission with menadione.

1-Methoxyphenazine methosulfate as a redox active agent

A study showed that the optimum concentrations for 1-methoxyphenazine methosulfate and lucigenin were 0.01 and 0.1 mM, respectively. Additional reactions were conducted in 50 mM Bis Tris Propane buffer, pH 9.5, 2.5mM glucose and 0.1 mM lucigenin with or without 0.01 mM 1-methoxyphenazine methosulfate. Light emission in the absence of 1-methoxyphenazine methosulfate was 590 and in its presence was 1670 demonstrating an enhancement by the redox agent.

Methylene blue as redox active agent

Optimization experiments conducted in 50 mM sodium pyrophosphate buffer, pH 9.9, showed that maximum light obtained with 0.01 mM methylene blue and 0.1 mM lucigenin. Reactions in 50 mM Bis Tris Propane buffer, pH 9.5, 0.1 mM lucigenin, 2.5 mM glucose gave 590 units of light in the absence of methylene blue and 4430 in the presence of methylene blue. A reaction conducted with methylene blue in 50 mM Bis Tris Propane buffer, pH 10.0, gave 26,000 units of light.

Plumbagin as redox active agent

Experiments conducted in 50 mM sodium pyrophosphate buffer, pH 9.0, obtained maximum light emission with 0.02 mM plumbagin and 0.2 mM lucigenin. Reactions in 50 mM Bis Tris Propane buffer, pH 9.5, with 0.2 mM lucigenin and without plumbagin gave 590 units of light compared to 1580 units in the presence of 0.02 mM plumbagin.

Example 6. Chemiluminescence in the presence of sodium cyanide as a metabolic inhibitor.

Various levels of sodium cyanide were included in reactions composed of 50 mM Bis Tris Propane buffer, pH 10.0, 2.5 mM glucose, 0.01 mM methylene blue, 0.2 mM lucigenin and 2.2 X 10^7 E. coli cells/reaction. Light emission was measured as in Example 5. Light emitted as a function of sodium cyanide concentration was as follows:

Sodium Cyanide (mM)	Light Emission (1 min.)
0.0	16,700
0.01	23, 900
0.02	30,400
0.05	19,300
0.1	15,400
0.2	11,900

These data show that 0.01 to 0.05 mM sodium cyanide increases light emission.

Example 7. Azure C as redox active agent.

A 200 µL reaction mixture containing 250 mM Bis Tris Propane buffer, pH 10.0, 0.5 mM lucigenin, 2.5 mM glucose and 80 µM Azure C was prepared at ambient temperature and light emission was integrated for one minute with the BioOrbit luminometer. The integrated background light was 117 units and the emission increased to 3850 units when about 5 X 10^5 E. coli cells (barn yard isolate) were added to the mixture. Another reaction mixture was prepared without Azure C and light emission without cells was 103 units and with cells was 352 units.

The present invention is disclosed by the description and examples presented above. However, many modifications and variations are possible within the scope and spirit of the invention.

WHAT IS CLAIMED IS:

1. A chemiluminescent reagent composition useful for the detection or characterization of viable microorganisms, comprising:

a. a substrate capable of metabolism by viable microorganisms of interest to produce reducing equivalents; and

b. a chemiluminescent probe.

2. The chemiluminescent composition of claim 1, wherein the chemiluminescent probe is lucigenin.

3. The composition of claim 2 wherein the substrate is chosen from glucose, citrate or ethanol or mixtures thereof.

4. The chemiluminescent composition of claim 2 which additionally includes a redox active agent.

5. The chemiluminescent composition of Claim 4 wherein the redox active agent is selected from the group consisting of Azure C and methylene blue.

6. The chemiluminescent composition of claim 1, which additionally includes a redox active agent capable of reacting with the reducing equivalents produced and the chemiluminescent probe is luminol.

7. The composition of claim 6 wherein the substrate is chosen from the group consisting of glucose, citrate or ethanol or mixtures thereof.

8. The chemiluminescent composition of claim 7, wherein the redox active agent is selected from the group consisting of paraquat, juglone and plumbagin.

9. The chemiluminescent composition of claim 1, wherein the microorganism of interest is a bacterium and the substrate is selected from the group consisting of glucose, citrate, and mixtures thereof.

10. The chemiluminescent composition of claim 1, wherein the microorganism of interest is a yeast and the substrate is ethanol.

11. A method of detecting and characterizing viable microorganisms, comprising the steps of:

a. contacting a sample suspected of contamination with microorganisms of interest with a chemiluminescent reagent composition comprising a substrate capable of being metabolized by the microorganism of interest and a chemiluminescent probe; and

b. measuring the light emitted.

12. The method of claim 11 wherein the sample is pretreated to provide a concentrate containing microorganisms of interest and the concentrate is contacted with the reagent composition.

13. The method of claim 12 wherein the pretreatment is selected from filtering or centrifuging the sample and the filtered cells or cell pellet obtained by the respective pretreatment method is contacted with the reagent composition.

14. A method of detecting and characterizing viable microorganisms, comprising the steps of:

a. contacting a sample suspected of contamination with microorganisms of interest with a chemiluminiscent reagent composition comprising a substrate capable of

being metabolized by the microorganism of interest, a redox active agent and a chemiluminiscent probe; and

b. measuring the light emitted.

15. A method of characterizing microorganisms of interest comprising the steps of:

a. contacting a sample suspected of contamination with microorganisms of interest with a chemiluminescent reagent composition comprising a substrate characteristic for the metabolism of the microorganism capable of being metabolized by the microorganism, a redox agent and a chemiluminescent probe; and

b. measuring the light emitted.

16. The method of claim 15 wherein the sample is pretreated to provide a concentrate containing microorganisms of interest and the concentrate is contacted with the reagent composition.

17. The method of claim 16 wherein the pretreatment is selected from filtering or centrifuging the sample and the filtered cells or cell pellet obtained by the respective pretreatment method is contacted with the reagent composition.

18. A method of detecting sensitivity to antibiotics, comprising the steps of:

a. contacting a sample containg microorganisms of interest with an antibiotic of interest and a chemiluminescent reagent composition comprising a substrate capable of being metabolized by the microorganism of interest and a chemiluminescent probe; and,

b. measuring any chemiluminescent light produced.

19. A method of detecting a toxicant, comprising the steps of:

a. contacting a sample suspected of containing a toxicant with a microorganism capable of being affected by a toxicant and a chemiluminescent reagent composition comprising a substrate capable of being metabolized by the microorganism and a chemiluminescent probe; and

b. measuring any chemiluminescent light produced.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/01869

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C 12 Q 1/02; C 12 Q 1/00
US CL :435/29; 435/7.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29; 435/7.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS: (Author: CARRICO, RJ); APS search terms: LUMINOL or LUCIGENIN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Archives of Biochemistry and Biophysics, Volume 196, No. 2, "Intracellular Production of Superoxide Radical and of Hydrogen Peroxide by Redox Active Compounds," issued September 1979, H.M. Hassan et al.	1-19
X, P	US,A, 5,084,381 (Akimoto et al) 28 January 1992, "Assay Method for Detecting Hydrogen Peroxide," see entire document	1-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 JUNE 1992	17 JUL 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JANE WILLIAMS Telephone No. (703) 308-0447

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